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FINAL REPORT

Development of an Assay Based on the Effects of PGB_x on the Isolated Perfused Rat Heart & Rat Skeletal Muscle

by

Carl E. Aronson, Ph.D.

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University of Pennsylvania School of Veterinary Medicine
Laboratories of Pharmacology & Toxicology
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Disophenol	Isoproterenol	Spontaneous heart rate

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

PART I

1. PGB_x at high concentrations ($\geq$500 ng/ml) depressed isometric systolic tension and coronary flow and decreased tissue concentrations of ATP and creatine phosphate.

20. (continued)

2. At 1000 ng/ml, PGB_x produced similar effects, but in addition, it markedly enhanced cardiac glycogen utilization, increased tissue concentrations of glucose-1-phosphate and glucose-6-phosphate and caused a significant increase in diastolic tension after being perfused for 60 minutes at this concentration.

PART II

1. In well-oxygenated perfused rat hearts, PGB_x had no effect on disophenol induced alterations in spontaneous heart rate, but did appear to prevent the increase in coronary flow caused by disophenol.

2. Isometric systolic tension decreased significantly in PGB_x-disophenol treated hearts but not in those receiving disophenol alone. Diastolic tension was increased in hearts which received PGB_x and disophenol simultaneously.

3. Disophenol caused alterations in glycogen and adenine nucleotide concentrations which were uninfluenced by PGB_x. The amount of ADP in the tissue, however, was lower in those hearts which received both PGB_x and disophenol. Phosphorylase activity and the lactate present in coronary effluent was the same in both groups.

PART III

1. Bolus injections of PGB_x (20-200 μg) did not alter significantly the mechanical and biochemical activity of isolated rat hearts perfused by a modified Langendorff technique.

2. Isoproternol caused a dose dependent increase in the conversion of phosphorylase *b* to *a* and a similar rise in isometric systolic tension, but PGB_x (100 ng/ml) did not alter the nature or magnitude of these isoproterenol-induced changes in cardiac performance.

3. PGB_x does not appear to release endogenous catecholamines from their storage sites in the heart or to enhance the sensitivity of *Beta* adrenergic receptors in this organ to isoproterenol.

PART IV

1. Under an environment of 100% O_2, PGB_x caused a significant dose dependent decrease in glucose uptake from the incubation medium by skeletal muscle, using the isolated rat diaphragm as a model system. Significant differences, however, were not observed at concentrations of PGB_x below 200 μg/flask. The glycogen content of the tissues examined was not altered by the presence of PGB_x in the incubation mixture at any of the concentrations studied.

2. When the experiments were repeated under room air, no significant effect of PGB_x on glucose uptake utilization or glycogen concentration could be detected.

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PART I

INTRODUCTION

PGB_x, a polymeric base-catalyzed derivative of 9,15-diketoprostaglandin B that is not to be confused with PGX (PGI_2 or prostocyclin) (Gryiewski *et al*, 1976; Johnson *et al*, 1976; Lefer *et al*, 1978), was first described and shown *in vitro* by Polis *et al* (1973) to reverse degenerative changes in mitochondria. In their system, addition of PGB_x restored oxidative phosphorylation and ATP values to normal in aged mitochondria (prepared from rat liver) whereas the untreated mitochondria showed further deterioration. These findings have since been confirmed by Devlin (1978). The ability of PGB_x to restore "normal" biochemical function in damaged mitochondria appears unique, and the drug shows promise of being of value in situations such as myocardial infarction, shock and acceleration stress where hypoxia of critical tissues and mitochondrial damage may possibly occur.

In monkeys, PGB_x had a protective effect against the mortality caused by coronary ligation and subsequent ventricular fibrillation, and mitochondria from infarcted areas of heart in PGB_x-treated animals showed minimal degenerative changes compared to those from untreated controls when examined by transmission electron microscopy (Riley *et al*, 1974; Angelokos *et al*, 1977).

Since little is known about the effects of the drug on normal tissue and its protective/stimulating effects on mitochondria appear to occur only in degenerating preparations (Riley *et al*, 1974), it was decided to investigate the effect of PGB_x on the isolated perfused rat heart, an *in vitro* system utilizing an intact organ (Aronson and Serlick, 1976, 1977a,b). The experiments reported herein were designed to determine direct effects of the drug on the heart in a system capable of detecting simultaneously, alterations in mechanical, electrical and biochemical activity.

MATERIALS AND METHODS

Surgical and Perfusion Methods

Wistar male albino rats (approximately 220-250 g) given Purina Lab Chow and water *ad libitum* were used in this study. The animals were sacrificed by decapitation and perfused by the Langendorff technique with Krebs-Ringer bicarbonate buffer (K-R buffer) as previously described (Aronson and Serlick 1976, 1977a).

Analytical Methods

Hearts selected for biochemical analysis were rapidly frozen in place on the perfusion apparatus by clamping the tissue with Wollenberger tongs which had been pre-cooled in liquid nitrogen (Wollenberger *et al*, 1960). The methods used to determine tissue metabolite concentrations and enzymatic activity have been reported in an earlier publication (Aronson and Serlick 1976).

Electrocardiographic Recording Techniques

Wick type electrodes were used to record electrical activity from the surface of the heart as previously described (Aronson and Serlick, 1977a, Aronson and Hanno, 1978). Heart rate, PR and QT intervals were determined directly from the original tracings according to previously established guidelines, and the QT interval was corrected for variability in heart rate (Aronson and Serlick, 1977b).

Statistical Methods

The standard error of the mean for each group was calculated and the data were examined by either the paired variate or independent t test (Ipsen and Feigl, 1970).

Drugs

The PGB_x used in these studies was synthesized and supplied by the Biochemistry Laboratory of the Naval Air Development Center, Warminster, Pennsylvania. PGB_x was made available to us in powder form as the sodium salt, and all dosages were calculated and expressed as such. The molecular weight of material which we used (Preparation #25) was taken to be 2150. All dilutions of the drug, prior to its addition to the perfusion medium, were made with physiological saline (PSS, 0.9% NaCl). PGB_x was considered stable under the storage conditions which we employed and those of our perfusion system (Polis, 1978).

RESULTS

Effect of PGB_x on Mechanical Activity in the Isolated Perfused Rat Heart

In hearts perfused with control (drug-free) medium for 60 minutes after the initial 15 minute equilibration period, diastolic tension declined slowly throughout the course of the experiment (Table 1-1). Spontaneous heart rate decreased simultaneously in this same population of hearts whereas coronary flow remained relatively constant. Isometric systolic tension developed by control hearts increased during the first 30 minutes of perfusion, but by 60 minutes returned to a value not significantly different from control.

When PGB_x was added to the medium and hearts were perfused at different concentrations (10, 100, 500 and 1000 ng/ml), alterations in coronary flow, isometric systolic tension and diastolic tension were observed. The greatest effects occured at 500 and 1000 ng/ml respectively. At these concentrations, coronary flow and isometric systolic tension were reduced significantly whereas comparable control hearts showed no such changes. Diastolic tension decreased

initially at 15 minutes, but subsequently increased to a value considerably above its initial value in PGB_x (1000 ng/ml)-treated hearts. This increase showed statistical significance only at the highest dose, whereas at lower doses, the responses more closely resembled those which occurred in the control hearts.

Isometric systolic tension decreased in each group of hearts perfused with PGB_x-containing medium (Table 1-1), and at the highest dose (1000 ng/ml), was approximately 30% its initial control value compared to 90% in the control group.

Coronary flow (Table 1-1) remained relatively constant at low doses of PGB_x (10 and 100 ng/ml), but at higher concentrations (500 and 1000 ng/ml), it decreased to 57% and 41% of control, respectively. In control hearts, on the other hand, flow was unchanged.

Spontaneous heart rate was variable, and while decreases occurred in PGB_x-perfused hearts, a similar decrease took place in the control group (Table 1-1). It is difficult, therefore, to ascribe a negative chronotropic action to PGB_x in view of the changes observed in the control group.

Effect of PGB_x on Electrical Activity in the Isolated Perfused Rat Heart

PGB_x had no effect on the electrical activity of the perfused heart at two of the four concentrations studied in this system (Table 1-2). At 500 and 1000 ng/ml, however, an intermittant increase in the duration of the QT interval was observed, but by 60 minutes the values returned to within normal limits. The PR interval showed a transient increase at 15 and 30 minutes at 1000 ng/ml, but once again the prolongation was not significant at 45 and 60 minutes respectively.

Recordings of electrical activity showed little deviation from the pattern normally observed and no serious arrhythmias were observed consistently and/or

ascribed to PGB_x. In one heart, however, AV block occurred briefly at 1000 ng/ml (Figure 1-1) after 60 minutes of perfusion, but it reverted spontaneously to a normal sinus rhythm.

Effect of PGB_x on Metabolite Concentrations in the Isolated Perfused Rat Heart

At 1000 ng/ml (Table 1-3), there was a marked decrease in the concentration of tissue glycogen in the heart accompanied by an increase in the amount of D-glucose-1-PO_4 and D-glucose-6-PO_4 present in the same samples. Adenosine-5'-triphosphate (ATP), total adenine nucleotides (AMP+ADP+ATP), and creatine phosphate were also significantly reduced. The profile was similar to 500 ng/ml, but glycogen and D-glucose-1-PO_4 concentrations were not altered significantly compared to hearts perfused only with drug-free medium.

Phosphorylase *a* activity was not elevated from control (14.1 $\pm$ 1.2%) by PGB_x at the concentrations studied after one hour of perfusion with the drug, although the diminished glycogen concentration which occurred at 1000 ng/ml (Table 1-3) suggests that enhanced activity of this system probably occurred earlier in time during the course of the experiment.

The amount of lactate found in coronary effluents was relatively constant in control hearts and at the lower doses of PGB_x, but at 500 and 1000 ng/ml was considerably elevated above control values by 60 minutes.

DISCUSSION

The ability of PGB_x to restore oxidative phosphorylation and enhance ATP synthesis in aged and/or damaged mitochondria is unique (Polis *et al*, 1973; Devlin, 1978), and its lack of effect on the metabolism of normal mitochondria suggests

that perhaps it does not gain access to target sites under "normal" conditions. Recent work, however, has shown PGB_x to have a biphasic nature, that is to stimulate aged mitochondria at lower doses and to inhibit their metabolism at higher concentrations (Devlin, 1978).

Our findings in the isolated perfused rat heart are consistant, in this regard, with those reported in mitochondria, since we found the drug had no effect in normal hearts at low concentrations (10 and 100 ng/ml), but appeared to depress the heart at higher concentrations (500 and 1000 ng/ml). At the latter concentrations, its biochemical effects closely resembled those produced in isolated perfused rat hearts by disophenol and bunamidine, veterinary anthelmintics (Aronson and Serlick, 1977b; Aronson and Hanno, 1978), and di-2-ethylhexyl phthalate (DEHP), a plasticizer (Aronson *et al.*, 1978). Each of these agents markedly enhanced glycogen utilization and lowered the concentration of ATP and total adenine nucleotides (ATP+ADP+AMP) in the heart in addition to modifying similarly the concentrations of several other metabolites.

The effects on mechanical activity produced by PGB_x at 1000 ng/ml are similar to those caused by disophenol, bunamidine and DEHP in that these drugs depressed spontaneous heart rate, coronary flow and isometric systolic tension while they also increased diastolic tension at high concentrations. In aged mitochondria from rat liver, Polis (1977) demonstrated that disophenol inhibited this system in a manner analogous to 2,4-dinitrophenol, and PGB_x produced a dose dependent reversal of this inhibition when added to the system. The specific site and mechanism of this antagonism between disophenol and PGB_x in mitochondria remains unclear. However, since both drugs depressed the heart and produced similar biochemical effects in our system, we plan, in future experiments, to determine whether and how they interact in the isolated perfused rat heart. Our

objective will be to determine whether PGB_x can effectively antagonize and/or protect against disophenol-induced alterations in mechanical, electrical and biochemical functions.

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TABLE 1-1

EFFECT OF PGB_x ON MECHANICAL ACTIVITY OF THE ISOLATED PERFUSED RAT HEART[a]

Drug	Concentration[b]	N[c]	Time	Spontaneous Heart Rate (bpm)	Coronary Flow (ml/min)	Isometric Systolic Tension (g)	Diastolic Tension (g)
None	-	5	0	246 ± 13.2	9.3 ± 1.0	15.3 ± 0.9	4.6 ± 0.2
			15	220 ± 13.6[d]	9.2 ± 1.6	17.5 ± 1.1[d]	4.2 ± 0.1[d]
			30	218 ± 17.5[d]	9.4 ± 1.3	17.3 ± 1.1[d]	3.8 ± 0.2[d]
			45	218 ± 17.4[d]	10.2 ± 1.2	16.1 ± 0.7	3.6 ± 0.3[d]
			60	203 ± 21.9[d]	9.9 ± 1.4	13.7 ± 0.7	3.2 ± 0.3[d]
PGB_x	10 ng/ml	5	0	290 ± 16.1	7.6 ± 0.4	15.9 ± 0.5	5.0 ± 0.2
			15	290 ± 19.3	7.4 ± 0.4	15.8 ± 0.7	4.5 ± 0.2[d]
			30	284 ± 15.6	7.5 ± 0.5	15.2 ± 0.7	4.1 ± 0.1[d]
			45	274 ± 18.1	7.7 ± 0.4	14.7 ± 0.8	4.0 ± 0.1[d]
			60	272 ± 17.4	7.5 ± 0.8	13.4 ± 0.7[d]	3.9 ± 0.1[d]
PGB_x	100 ng/ml	5	0	257 ± 13.3	7.4 ± 0.5	14.8 ± 1.3	4.8 ± 0.1
			15	243 ± 9.1	7.0 ± 0.5	13.4 ± 1.7	4.3 ± 0.4
			30	231 ± 12.3[d]	7.9 ± 0.7	13.5 ± 0.8	4.1 ± 0.4
			45	236 ± 12.1[d]	7.0 ± 0.5	12.4 ± 0.8[d]	4.1 ± 0.4
			60	226 ± 10.9[d]	6.7 ± 0.5	10.4 ± 1.0[d]	4.1 ± 0.4
PGB_x	500 ng/ml	7	0	255 ± 9.2	7.5 ± 0.9	13.9 ± 0.7	4.7 ± 0.2
			15	234 ± 12.8[d]	7.5 ± 1.0	14.4 ± 0.3	4.2 ± 0.2[d]
			30	231 ± 14.6	6.3 ± 0.7	11.6 ± 1.2	4.3 ± 0.3
			45	213 ± 17.4[d]	5.3 ± 0.6[d]	8.7 ± 1.5[d]	4.8 ± 0.4
			60	198 ± 18.8[d]	4.3 ± 0.6[d]	6.8 ± 1.3[d]	5.1 ± 0.4
PGB_x	1000 ng/ml	5	0	244 ± 9.8	7.3 ± 0.8	16.9 ± 1.3	4.9 ± 0.1
			15	244 ± 13.7	6.8 ± 0.2	16.0 ± 1.0	4.3 ± 0.1[d]
			30	221 ± 14.6[d]	5.0 ± 0.3	11.5 ± 2.0	4.8 ± 0.3
			45	205 ± 22.5	3.9 ± 0.5	7.2 ± 1.0[d]	5.6 ± 0.3
			60	193 ± 19.1[d]	3.0 ± 0.3[d]	5.0 ± 0.4[d]	6.4 ± 0.1[d]

[a] Hearts obtained from normal male rats (220-250 g).
[b] PGB_x calculated and expressed as the sodium salt. Hearts were perfused with control or PGB_x-containing medium for 60 minutes after the initial 15 minute equilibration period.
[c] Number of hearts in each group.
[d] Significant ($P<0.05$) compared to 0 perfusion time within each group by paired variate t test.

TABLE 1-2

EFFECT OF PGB_x ON ELECTRICAL ACTIVITY OF THE ISOLATED PERFUSED RAT HEART[a]

Drug	Concentration[b]	N[c]	Time	PR (ms)	QT (ms)	QTc[d]
None	-	5	0	45 ± 1.4	81 ± 3.3	5.16 ± 0.23
			15	44 ± 1.8	83 ± 2.0	5.01 ± 0.16
			30	46 ± 1.9	84 ± 2.4	5.03 ± 0.11
			45	45 ± 2.1	84 ± 1.8	5.01 ± 0.14
			60	44 ± 1.6	84 ± 2.4	4.83 ± 0.13
PGB_x	10 ng/ml	5	0	44 ± 2.0	76 ± 1.9	4.84 ± 0.30
			15	44 ± 1.8	77 ± 2.0	4.93 ± 0.33
			30	45 ± 1.5	74 ± 2.5	4.85 ± 0.33
			45	44 ± 1.6	75 ± 2.2	4.73 ± 0.30
			60	44 ± 1.8	71 ± 3.3	4.76 ± 0.37
PGB_x	100 ng/ml	5	0	42 ± 1.0	70 ± 3.2	4.59 ± 0.16
			15	42 ± 1.0	70 ± 3.2	4.47 ± 0.16
			30	42 ± 1.0	72 ± 4.9	4.46 ± 0.21
			45	41 ± 0.8	73 ± 5.5	4.55 ± 0.26
			60	43 + 1.9	73 ± 5.5	4.45 ± 0.30
PGB_x	500 ng/ml	7	0	45 ± 1.5	79 ± 1.4	5.13 ± 1.13
			15	45 ± 1.1	83 ± 2.9	5.17 ± 0.22
			30	46 ± 1.1	84 ± 3.0	5.22 ± 0.25
			45	47 ± 0.9	86 ± 3.7	5.08 ± 0.31
			60	47 ± 1.4	86 ± 4.3	4.90 ± 0.33
PGB_x	1000 ng/ml	5	0	46 ± 1.9	78 ± 4.9	4.97 ± 0.24
			15	50 ± 1.9[e]	88 ± 8.6	5.55 ± 0.4[illegible]
			30	52 ± 2.6[e]	88 ± 7.3[e]	5.29 ± 0.32
			45	62 ± 9.9	84 ± 5.1	4.82 ± 0.15
			60	66 ± 11.6	84 ± 5.1	4.70 ± 0.21

[a] Hearts obtained from normal male rats (220-250 g).

[b] PGB_x calculated and expressed as the sodium salt. Hearts were perfused with control or PGB_x-containing medium for 60 minutes after the initial 15 minute equilibration peri[illegible]

[c] Number of hearts in each group.

[d] $QTc = \frac{QT\ (ms)}{\sqrt{R\text{-}R\ (ms)}}$

[e] Significant ($P \leq 0.05$) compared to 0 perfusion time within each group by paired variate t te[illegible]

TABLE 1-3

EFFECT OF PGB_x ON METABOLITE CONCENTRATIONS IN THE ISOLATED PERFUSED RAT HEART[a]

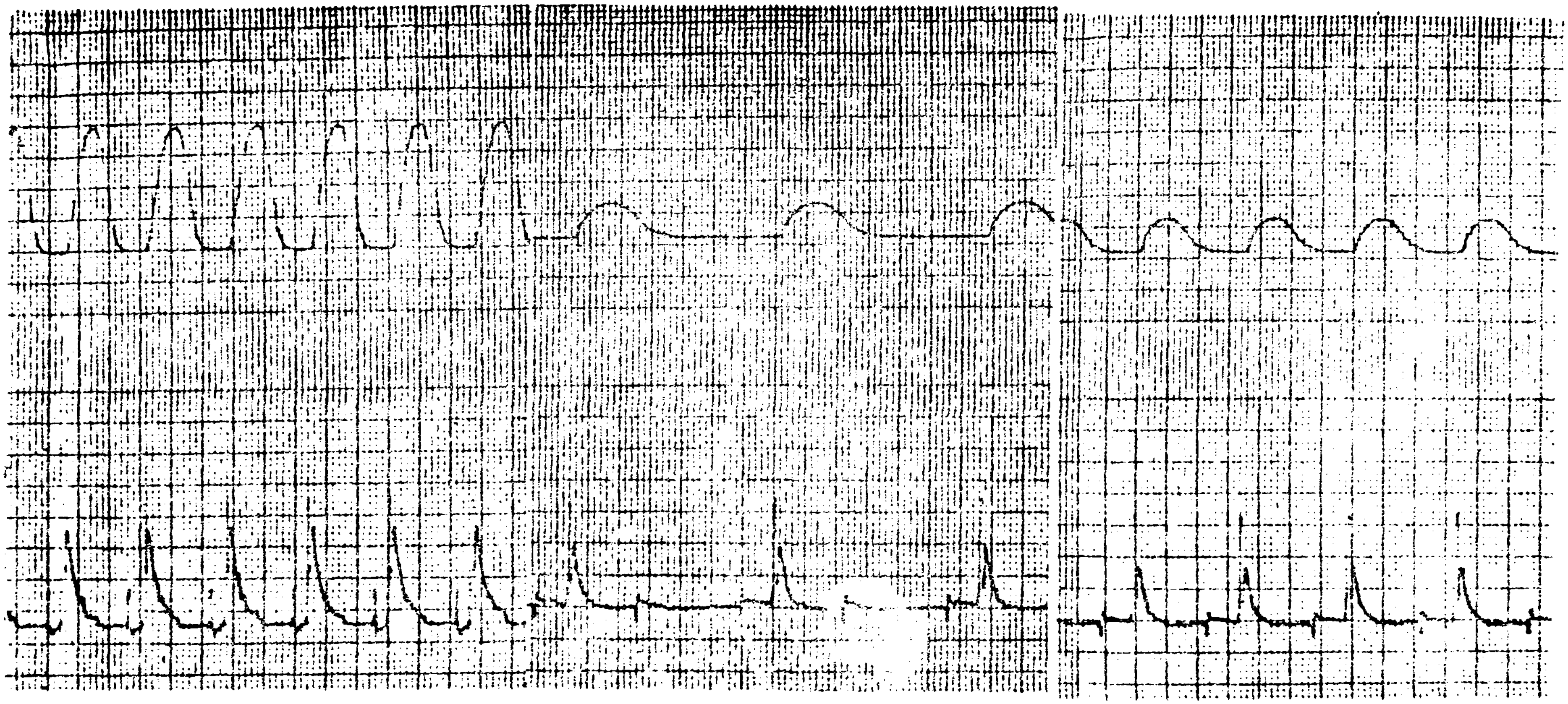
Metabolite	Concentration of PGB_x in Perfusion Medium[b]									
	N[c]	0 ng/ml μM/g ± SEM[d]	N[c]	10 ng/ml μM/g ± SEM[d]	N[c]	100 ng/ml μM/g ± SEM[d]	N[c]	500 ng/ml μM/g ± SEM[d]	N[c]	1000 ng/ml μM/g ± SEM[d]
Glycogen	5	13.51 ± 1.43	5	11.02 ± 1.08	5	12.65 ± 1.45	7	11.13 ± 1.74	5	6.92 ± 1.04[e]
D-Glucose-1-PO_4	5	0.0000 ± 0.0000	5	0.0000 ± 0.0000	5	0.0000 ± 0.0000	7	0.0099 ± 0.0057	5	0.0260 ± 0.095[e]
D-Glucose-6-PO_4	5	0.0200 ± 0.0034	5	0.0191 ± 0.0068	5	0.0346 ± 0.0087	7	0.0690 ± 0.0173[e]	5	0.0976 ± 0.0116[e]
D-Fructose-6-PO_4	5	0.0083 ± 0.0051	5	0.0120 ± 0.0042	5	0.0120 ± 0.0068	7	0.0289 ± 0.0169	5	0.0156 ± 0.0064
D-Fructose-1,6-di PO_4	5	0.0360 ± 0.0034	5	0.0332 ± 0.0069	5	0.0243 ± 0.0067	7	0.0391 ± 0.0047	5	0.0487 ± 0.0158
Dihydroxyacetone PO_4	5	0.1210 ± 0.0103	5	0.1044 ± 0.0115	5	0.0577 ± 0.0158	7	0.1197 ± 0.0114	5	0.1026 ± 0.0211
D-Glyceraldehyde-3-PO_4	5	0.0648 ± 0.0144	5	0.0601 ± 0.0101	5	0.0546 ± 0.0119	7	0.1038 ± 0.0219	5	0.1185 ± 0.0233
L-(-)-Glycerol-1-PO_4	5	0.2366 ± 0.0409	5	0.2939 ± 0.0504	5	0.3661 ± 0.0580	7	0.2878 ± 0.0471	5	0.3863 ± 0.0998
Pyruvate	5	0.1066 ± 0.0542	5	0.1574 ± 0.0527	5	0.1142 ± 0.0454	7	0.1509 ± 0.0295	5	0.1680 ± 0.0481
L-(+)-Lactate	5	0.7330 ± 0.1337	5	0.4657 ± 0.0528	5	0.8825 ± 0.2592	7	1.2970 ± 0.3905	5	1.3578 ± 0.2934
Adenosine-5'-tri PO_4	5	2.4805 ± 0.2484	5	2.2926 ± 0.0778	5	2.1160 ± 0.1199	7	1.3287 ± 0.1889[e]	5	1.1883 ± 0.0814[e]
Adenosine-5'-di PO_4	5	0.2890 ± 0.0546	5	0.3676 ± 0.0617	5	0.3015 ± 0.0356	7	0.2411 ± 0.0380	5	0.2531 ± 0.0473
Adenosine-5'-mono PO_4	5	0.0715 ± 0.0110	5	0.0955 ± 0.0173	5	0.0953 ± 0.0108	7	0.0714 ± 0.0141	5	0.0470 ± 0.0141
Total Adenine Nucleotides	5	2.8411 ± 0.2760	5	2.7557 ± 0.0832	5	2.5128 ± 0.1143	7	1.6412 ± 0.1975[e]	5	1.4885 ± 0.0922[e]
Creatine-PO_4	5	2.4874 ± 0.2334	5	2.2272 ± 0.1820	5	2.0056 ± 0.2437	7	1.6413 ± 0.1550[e]	5	1.5881 ± 0.1446[e]

[a] Hearts obtained from normal male rats (220-250 g).
[b] PGB_x calculated and expressed as the sodium salt. Hearts were perfused with control or PGB_x-containing medium for 60 minutes after the initial 15 minute equilibration period.
[c] Number of hearts in each group.
[d] Expressed per gram of tissue (wet weight).
[e] Significant ($P \leq 0.05$) compared to control (0 drug concentration) group by an independent t test.

FIGURE LEGEND

Figure 1-1

These recordings were made from a heart perfused with Krebs-Ringer Bicarbonate buffer containing PGB_x (1000 ng/ml). Isometric systolic tension (1 g/mm) is shown in the upper part of each tracing, while electrical activity (2 mV/cm) is shown below. Paper speed was 50 mm/sec. The first or 0 time recording (A) was made at the end of the 15-minute equilibration period with drug-free buffer, after which the heart was perfused with medium containing PGB_x. After 60 minutes of perfusion with PGB_x, the heart developed a transient 2:1 AV block (B) which reverted spontaneously to a normal sinus rhythm (C).



(A) (B) (C)

FIGURE 1-1

PART II

INTRODUCTION

In Part I of this report, data were presented which showed that PGB_x produced a dose related depression of isometric systolic tension and coronary flow in the isolated perfused rat heart. PGB_x also altered glycogen metabolism and ATP production in these same hearts.

Disophenol, an anthelmintic drug, produced similar effects in this preparation (Aronson and Serlick, 1977), but Polis (1977, Personal communication) found that PGB_x antagonized the inhibitory action of disophenol in aged mitochondria (prepared from liver). Eichel (1979, Personal communication), in related experiments, confirmed the earlier work of Polis (1977, Personal communication) in aged mitochondria which showed disophenol to be a true inhibitor of oxidative phosphorylation. He found, however, that PGB_x was limited in its ability to counteract and reverse the disophenol-induced depression of mitochondrial oxidative phosphorylation in preparations of aged mitochondria. Disophenol also inhibited oxidative phosphorylation in fresh preparations of mitochondria, but had a greater effect in the aged preparation (Eichel, 1979, Personal communication).

The studies presented in this section of the report (Part II) were conducted to determine whether pre-perfusion with PGB_x (100 ng/ml) would affect the deleterious action of disophenol on well oxygenated normal hearts.

MATERIALS AND METHODS

The surgical, perfusion, rapid freezing and analytical techniques and statistical methods described and referenced in Part I of this report were used

also in the experiments reported in Part II.

Disophenol (40 μg/dose) was injected as a bolus into the flow of perfusion fluid entering the heart at exactly 5 minutes and 10 minutes after the initial 15 minute equilibration period with drug-free K-R buffer. In those hearts perfused with PGB_x-containing medium (100 ng/ml), PGB_x began entering the heart immediately upon the conclusion of the 15 minute equilibration period and continued through the course of the experiment. These hearts, therefore, were pre-perfused with PGB_x for exactly 5 minutes before receiving their first dose of disophenol.

The 100 ng/ml concentration of PGB_x was selected because it produced no evidence of electrical or biochemical disturbance in the heart. The 40 μg dose of disophenol, administered as a bolus, was determined experimentally as one which would cause an almost immediate depression upon injection, but generally not cardiac arrest.

The disophenol (2,6-diiodo-4-nitrophenol, D.N.P.®) powder used in these studies was a gift from the American Cyanamid Company, Princeton, New Jersey. It was placed in solution with physiological saline (0.9% NaCl, PSS) and a drop of NaOH was added to facilitate solubility, after which the pH was adjusted to neutral before use.

RESULTS

Effect of PGB_x and Disophenol on Mechanical Activity of the Isolated Perfused Rat Heart

Hearts perfused with PGB_x (100 ng/ml) for 15 minutes showed no significant alterations in isometric systolic tension, coronary flow or diastolic tension compared to control hearts perfused with PGB_x-free medium. In both groups,

spontaneous heart rate declined significantly, but only the control group showed a decrease in resting diastolic tension (Table 2-1).

When disophenol was injected as a bolus, heart rate, isometric systolic tension and diastolic tension showed no significant change, but coronary flow Increased above control values briefly before returning to normal (Table 2-1). Hearts perfused with PGB_x-containing buffer that also received a bolus injection of disophenol showed a marked decrease in isometric systolic tension accompanied by a simultaneous elevation in diastolic tension while coronary flow, in contrast to hearts receiving disophenol alone, was unaltered when both drugs were combined.

Effects on Biochemical Activity

Perfusion with PGB_x (100 ng/ml)-containing medium lowered the amount of adenosine-5-triphosphate (ATP) (Table 2-2) in the tissue, but had no other effects on the metabolites which we measured. Disophenol, on the other hand, reduced significantly the concentration of glycogen, ATP, adenosine-5'-monophosphate (AMP) and total adenine nucleotides, but adenosine-5'-diphosphate (ADP) showed no change. When disophenol was administered to PGB_x-perfused hearts, tissue concentrations of glycogen, ATP, ADP, AMP and total nucleotides were significantly lower than those observed in control hearts, but with the exception of ADP (which was lower), they did not differ significantly from the values for the individual metabolites in the group receiving only disophenol. By inspection alone, however, the values in the PGB_x and disophenol combination group "appeared" lower than those in the disophenol group.

Measurements of the lactate present in the coronary effluent samples (Table 2-3) showed no difference in the response of the disophenol group compared to hearts treated with disophenol in the presence of PGB_x. Phosphorylase *a*

activity was also the same in each of the groups examined (Table 2-4).

DISCUSSION

The ability of PGB_x to prevent 2,4-dinitrophenol-induced inhibition of phosphorylation in aged preparations of liver mitochondria (Polis *et al*, 1979) and to interfere similarly with the action of disophenol (Polis, 1977, Personal communication) has been clearly established. Polis *et al* (1979) have shown also that PGB_x effectively competed with Ca^{2+} and antagonized Ca^{2+}-induced inhibition of mitochondrial phosphorylation. At low concentrations of 2,4-dinitrophenol, however, the actions of PGB_x and 2,4-dinitrophenol appeared to be synergistic in the mitochondrial system, since the degree of phosphorylation that occurred with the combination was greater than that which took place in the presence of PGB_x alone (Polis *et al*, 1979).

To date, the site(s) of action of PGB_x has(have) not been firmly established, but Devlin (1978, 1980, Personal communication) demonstrated also that PGB_x, in aged mitochondria, had a reproducible biphasic dose-related ability to either stimulate or inhibit oxidative phosphorylation, a finding consistant with our observation (Aronson & Guerrero, 1979) that PGB_x at higher doses, inhibited mechanical and biochemical activity in the well-oxygenated isolated perfused rat heart, whereas at lower concentrations, it had no measurable stimulatory effect on this preparation.

The data presented in this paper provides no evidence to support the thesis that disophenol and PGB_x are mutually antagonistic in the well-oxygenated isolated perfused rat heart. The data in Table 2-2 suggest, but do not prove, that both drugs may somehow actually be acting in concert to promote glycogen breakdown and to diminish ATP concentrations in our preparation.

Recently work by Biemann (1978, 1979, Personal communication) has demonstrated that PGB_x (Preparation #25) may actually contain several active components, one stimulatory and at least one inhibitory. Findings by Devlin (1980, Personal communication) in isolated mitochondria prepared from liver tend to confirm this observation, and there appears to be a delicate dose-related balance between the stimulatory and excitatory components which react differently in various biological systems. In the well oxygenated perfused heart, for example, at higher dosages, the effect of the inhibitory component may account for the negative inotropic and biochemical effects which we observed (Aronson and Guerrero, 1979). Recent evidence (Devlin, 1980, Personal communication, Ohnishi & Devlin, 1979) suggest that PGB_x and perhaps discphenol, a 2,4-dinitrophenol-like compound, act as ionophores, and in so doing, dissipate the proton gradient across the mitochondrial membrane. It appears on the basis of their data, however, that this ionophore property may relate just to the inhibitory fraction of Preparation #25 and not its stimulatory component. PGB_x-induced decreases in F_1F_0-ATPase activity have been detected also in Devlin's mitochondrial preparation (Kreutter and Devlin, 1980). In fresh mitochondria, isolated from liver, they found that PGB_x abolished respiratory control by inhibiting state 3 and slightly stimulating state 4 respiration. ATP-driven Ca^{2+} transport was inhibited, but the fact that it did not adversely alter respiration-linked Ca^{2+} uptake or Ca^{2+}-stimulated respiration implied to these investigators that PGB_x did not inhibit the activity of the respiratory enzyme chain. To date, similar data is not available regarding the actions of PGB_x on analogous enzyme and transport systems in the heart. Consequently, we can only speculate that perhaps it may act in the heart as it does in the liver, by a mechanism similar to, but not identical with that of oligomycin (Kreutter & Devlin, 1980).

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TABLE 2-1

INTERACTIONS BETWEEN PGB_x AND DISOPHENOL RELATING TO MECHANICAL ACTIVITY OF ISOLATED PERFUSED RAT HEARTS[a]

Drug	Time Sequence	N[g]	Spontaneous Heart Rate	Coronary Flow	Isometric Systolic Tension	Diastolic Tension
None	A[d]	6	275 ± 25.4	6.8 ± 0.4	13.5 ± 1.2	4.7 ± 0.2
	B[e]	6	267 ± 21.7	6.4 ± 0.4[h]	14.1 ± 1.2	4.5 ± 0.2
	C[f]	6	246 ± 19.6[h]	7.1 ± 0.4	14.3 ± 1.3	4.1 ± 0.2[h]
PGB_x[b]	A[d]	5	275 ± 12.0	8.0 ± 0.4	15.7 ± 1.7	4.5 ± 0.1
	B[e]	5	271 ± 9.1	7.5 ± 0.2	15.8 ± 1.5	4.4 ± 0.3
	C[f]	5	251 ± 12.2[h]	8.1 ± 0.3	16.0 ± 1.4	4.0 ± 0.3
Disophenol[c]	A[d]	5	272 ± 16.0	7.4 ± 0.5	14.3 ± 0.9	4.5 ± 0.1
	B[e]	5	196 ± 50.5	10.0 ± 0.6[h]	8.7 ± 2.4	8.6 ± 1.9
	C[f]	5	198 ± 49.9	8.2 ± 0.6	11.4 ± 3.1	6.8 ± 2.1
PGB_x[b] +	A[d]	5	266 ± 5.5	8.4 ± 0.4	14.6 ± 0.7	4.7 ± 0.2
Disophenol[c]	B[e]	5	228 ± 27.4	8.6 ± 1.6	5.7 ± 2.4[h]	10.6 ± 1.3[h]
	C[f]	5	241 ± 10.7[h]	7.7 ± 0.3	10.4 ± 0.3	7.1 ± 1.3

[a] Hearts obtained from normal male rats (220-250 g).
[b] PGB_x calculated and expressed as the sodium salt. Concentration in K-R buffer = 100 ng/ml. Flow started at end of 15 minute equilibration period.
[c] Disophenol (40 μg) injected as a bolus into the flow of perfusion fluid entering the heart at 5 and 10 minutes after initial 15 minute equilibration period.
[d] A = 5 minutes after initial 15 minute equilibration period.
[e] B = 15 minutes after initial 15 minute equilibration period.
[f] C = 30 minutes after initial 15 minute equilibration period.
[g] Number of hearts in each group.
[h] Significant ($P \leq 0.05$) compared to A in each group by paired variate P test

TABLE 2-2

INTERACTIONS BETWEEN PGB_x AND DISOPHENOL RELATING TO METABOLITE CONCENTRATIONS IN ISOLATED PERFUSED RAT HEARTS[a]

Metabolite	Control[b]		PGB_x[b]		Disophenol[b]		PGB_x[b] + Disophenol[b]	
	N[c]	µM/g[d] ± SEM	N[c]	µM/g[d] ± SEM	N[c]	µM/g ± SEM	N[c]	µM/g ± SEM
Glycogen	6	18.07 ± 1.02	5	15.99 ± 0.57	5	12.40 ± 1.95[e]	5	9.70 ± 2.87[e]
D-Glucose-1-PO_4	6	0.0062 ± 0.0032	5	0.0019 ± 0.0014	5	0.0074 ± 0.0035	5	0.0037 ± 0.0024
L-(-)-Glycerol-1-PO_4	6	0.1140 ± 0.0244	5	0.1349 ± 0.1283	5	0.1442 ± 0.0489	5	0.1183 ± 0.0512
L-(+)-Lactate	6	0.4652 ± 0.0578	5	0.4864 ± 0.0572	5	0.3868 ± 0.1113	5	0.3961 ± 0.1567
Adenosine-5'-tri PO_4	6	2.9761 ± 0.1518	5	2.4550 ± 0.1640[e]	5	2.1872 ± 0.2528[e]	5	1.9491 ± 0.1956[e]
Adenosine-5'-di PO_4	6	0.5602 ± 0.0537	5	0.5567 ± 0.0329	5	0.4316 ± 0.0371	5	0.3036 ± 0.0342[e]
Adenosine-5'-mono PO_4	6	0.0510 ± 0.0050	5	0.0903 ± 0.0183[e]	5	0.1852 ± 0.0517[e]	5	0.1528 ± 0.0535[e]
Total Adenine Nucleotides	6	3.5872 ± 0.1803	5	3.1021 ± 0.1924	5	2.8040 ± 0.2413[e]	5	2.4055 ± 0.2028[e]
Creatine PO_4	6	2.2098 ± 0.1583	5	1.8774 ± 0.1410	5	2.3163 ± 0.1815	5	2.8400 ± 0.3337

[a]Hearts obtained from normal male rats (220-250 g).
[b]Hearts frozen 30 minutes after initial 15 minute equilibration period at the end of time sequence C as defined in footnotes to Table 1. Drugs, their concentrations and mode of administration are as stated in the footnotes to Table 1.
[c]Number of hearts in each group.
[d]Expressed per gram of tissue (wet weight).
[e]Significant ($P \leq 0.05$) compared to control by an independent t test.

TABLE 2-3

INTERACTIONS BETWEEN PGB_x AND DISOPHENOL RELATING TO LACTATE CONCENTRATIONS IN CORONARY EFFLUENTS FROM ISOLATED PERFUSED RAT HEARTS[a]

Drug[b]	Time Sequence[c]	N[d]	Lactate (μM/g/min)[e]
None	A	6	0.282 $\pm$ 0.028
	B	6	0.257 $\pm$ 0.027
	C	6	0.240 $\pm$ 0.030
PGB_x	A	5	0.287 $\pm$ 0.071
	B	5	0.235 $\pm$ 0.044
	C	5	0.218 $\pm$ 0.057[f]
Disophenol	A	5	0.272 $\pm$ 0.068
	B	5	0.409 $\pm$ 0.079
	C	5	0.306 $\pm$ 0.117
PGB_x + Disophenol[b]	A	5	0.265 $\pm$ 0.047
	B	5	1.006 $\pm$ 0.366
	C	5	0.225 $\pm$ 0.036

[a]Hearts obtained from normal male rats (220-250 g).
[b]Drugs, their concentrations and mode of administration are identical to those described in the footnotes to Table 1.
[c]Time sequences identical to those described in the footnotes to Table 1.
[d]Number of hearts in each group.
[e]Calculated on the basis of tissue wet weight
[f]Significant ($P \leq 0.05$) compared to A in each group by paired variate t test.

TABLE 2-4

EFFECTS OF INTERACTIONS BETWEEN PGB_x AND DISOPHENOL RELATING TO PHOSPHORYLASE a ACTIVITY IN ISOLATED PERFUSED RAT HEARTS[a]

Drug [b]	N[c]	% Phosphorylase a [d]
None	6	15.1 ± 1.63
PGB_x	5	15.3 ± 1.23[e]
Disophenol	5	14.1 ± 1.44[e]
PGB_x + Disophenol	5	13.7 ± 1.27[e]

[a]Hearts obtained from normal male rats (220-250 g).
[b]Hearts frozen 30 minutes after initial 15 minute equilibration period at the end of time sequence C as defined in footnotes to Table 1. Drugs, their concentrations and mode of administration are as stated in the footnotes to Table 1.
[c]Number of hearts in each group
[d]$\%\ \text{Phosphorylase } a = \frac{\text{Cori Units of phosphorylase a}}{\text{Total Cori Units (a+b)}} \times 100$
[e]Not significant ($P>0.05$) when compared to control (drug free) group by an independent t test.

PART III

INTRODUCTION

The experiments described in the first part of Part III were performed to ascertain whether bolus administration of PGB_x, in contrast to prolonged perfusion, might enhance cardiac mechanical and biochemical activity as suggested by the *in vivo* studies conducted in monkeys (Angelakos *et al*, 1977). We sought to determine whether PGB_x-induced cardiac stimulation would occur, and if so, whether it might be mediated by the release of endogenous catecholamines, or perhaps by an increase in the sensitivity of the myocardium to endogenous catecholamines.

In order to find out whether PGB_x altered the responsiveness of perfused rat hearts to catecholamines, the preparation was challenged, in the presence and absence of PGB_x in the perfusion medium, with graded doses of isoproterenol over a wide concentration range (2.5-100 ng), and the effects of the isoproterenol challenges on phosphorylase activity, isometric systolic tension development, and glycogen utilization were measured. Isoproterenol was selected as the agonist in our experiments because of its selectivity for *Beta* adrenergic receptors, and because it is not actively taken up by sympathetic postganglionic nerve terminals. Consequently, we sought to focus our attention on possible postsynaptic mechanisms for potentiation rather than those which might be mediated by a "cocaine-like" inhibition of neuronal presynaptic catecholamine uptake mechanisms.

MATERIALS AND METHODS

The surgical, perfusion, rapid freezing and analytical techniques described and referenced in Part I of this report were also used in the experiments reported in this section (Part III).

Each heart was equilibrated initially for 15 minutes with drug-free K-R buffer prior to beginning the experimental sequence. In the experiments in which PGB_x was injected as a bolus, the drug was administered in a volume of 0.2 ml of physiological saline solution (PSS, 0.9% NaCl) five minutes after completion of the 15 minute equilibration period. The bolus injection of PGB_x was followed immediately with 0.5 ml of K-R buffer in order to clear the cannula of drug and facilitate the delivery of PGB_x into the flow of K-R buffer entering the heart.

When isoproterenol was administered to the preparation, it was also injected in a 0.2 ml volume and followed by a K-R buffer washed as described above. In these experiments, however, the hearts were perfused with either drug-free or PGB_x-containing K-R buffer for exactly 15 minutes following the initial 15 minute equilibration period, prior to injecting the isoproterenol.

During the course of our experiments, wick type electrodes were used to record and monitor electrical activity from the surface of the heart as described previously (Aronson and Serlick, 1977a,b; Aronson and Hanno, 1978). While this data is not presented, it was used to verify the status and stability of the individual preparations.

The data were examined by analysis of variance (Snedicor and Cocran, 1967; Sokal and Rohlf, 1969; Winer, 1962).

The PGB_x used in the experiments reported in this section was obtained, prepared and stored as described in Part I. Isoproterenol solutions were prepared fresh and diluted in an appropriate manner with physiological saline immediately prior to each experiment.

RESULTS

When PGB_x was administered to isolated perfused hearts as a bolus, in dosages up to 200 μg, the drug did not significantly alter the mechanical or biochemical activity measured in this preparation.

In Table 3-1, phosphorylase activity was the same in the hearts which received the bolus of PGB_x as it was in those which received a bolus of saline of the same volume. The glycogen concentration in these same hearts also showed no variation from the control group (Table 3-2).

Although each group showed a small increase in isometric systolic tension after the injection sequences was completed, the changes were not significant (Table 3-3).

Administration of isoproterenol, as a bolus, caused a dose dependant increase in the conversion of phosphorylase *b* to *a* (Table 3-4) in hearts perfused with drug-free K-R buffer. When a second series of hearts were perfused in the same manner with PGB_x (100 ng/ml)-containing medium, an increase in phosphorylase a activity was also observed in response to the isoproterenol challenge. However, evaluation of the data by analysis of variance, determined that the responses over the entire dosage range of isoproterenol were the same in the PGB_x perfused and the control groups.

In the same population of hearts, isoproterenol and PGB_x had no effect on the concentration of glycogen in the tissues which we examined (Table 3-5).

Isometric systolic tension increased in response to isoproterenol over the dosage range examined, but PGB_x did not alter the magnitude of the response nor

was there a statistically significant interaction between PGB_x and the concentration of isoproterenol administered (Table 3-6).

DISCUSSION

When PGB_x was administered to isolated perfused rat hearts as a bolus, over a wide dosage range, the drug did not significantly alter their mechanical activity (Table 3-3) and we found no biochemical evidence of any PGB_x-induced sympathomimetic effects (Table 3-1 & 3-2). If PGB_x had exerted a direct stimulant action on *Beta* receptors in the heart, we would have expected to have observed a positive inotropic response which, in our preparation, would have been measured as an increase in isometric systolic tension. One would also have expected such an increase to have exhibited at least some degree of dose dependency. An indirect sympathomimetic response, mediated by a PGB_x-induced release of catecholamines from sympathetic post ganglionic nerve terminals in the heart, would also have caused a positive inotropic response in this preparation had it occurred.

The ability of the enzyme systems which promote the conversion of phosphorylase *b* to *a* in the heart to respond to endogenously released and exogenously administered catecholamines has been well documented (Haugaard and Hess, 1965; Horn *et al*, 1967; Aronson and Hess, 1967; Aronson *et al*, 1972; Aronson and Hanno, 1978). In our experiments, however, hearts which received bolus injections of PGB_x, ranging up to 200 μg/injection, showed no difference in phosphorylase a activity from that measured in control group which received a comparable volume of drug-free physiological saline. The glycogen content of the PGB_x-treated and the control hearts were also not significantly different (Table 3-2), probably

due to the relatively short duration of the perfusion procedure in these particular experiments.

In hearts challenged with bolus injections of isoproterenol (2.5-100 ng), both the conversion of phosphorylase *b* to *a* and the development of isometric systolic tension increased in a dose-dependent manner (Tables 3-4 & 3-6), but the presence of PGB_x (100 ng/ml) in the perfusion medium of one group of hearts had no effect on the magnitude of the isoproterenol-induced changes mentioned above. The analysis of variance also demonstrated there was not a significant relationship (interaction) between treatment with PGB_x and the dose of isoproterenol with which the hearts were challenged. The dose of PGB_x (100 ng/ml) used for perfusion in the isoproterenol-challenge experiments was selected for this study because our previous work had shown that this concentration of drug in the perfusion medium, although it depressed isometric systolic tension in prolonged experiments (60 minutes), did not adversely alter cardiac metabolism (Aronson and Guerrero, 1979).

Since our experiments did not demonstrate a PGB_x-induced enhancement of the hearts ability to respond to mechanical and biochemical changes produced by isoproterenol, we concluded that PGB_x, under our conditions, had no direct effect upon *Beta* receptors in the heart. Furthermore, it would, therefore, be reasonable to speculate that perhaps the augmentation of catecholamine effects observed *in vivo* by Angelakos *et al* (1977) was produced by some mechanism other than direct *Beta* receptor stimulation by PGB_x or by a PGB_x-induced enhancement of the sensitivity of *Beta* receptors to catecholamines. Since the studies reported by Angelakos *et al* (1977) were conducted *in vivo*, it is also realistic to consider that perhaps a metabolite of PGB_x, rather than the parent compound, was responsible for the effects that they observed.

While we cannot rule out the possibility that PGB_x may facilitate the release of endogenous catecholamines or some other pressor substances under appropriate conditions *in vivo*, we have no evidence that it promotes catecholamine release directly in well oxygenated isolated perfused rat hearts.

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TABLE 3-1

EFFECTS OF BOLUS ADMINISTRATION OF PGB_x ON PHOSPHORYLASE ACTIVITY IN ISOLATED PERFUSED RAT HEARTS[a]

Drug	Dose[d]	N[e]	% Phosphorylase a[f]
PSS[b]	-	6	14.0 ± 1.05
PGB_x[c]	20 μg	7	14.5 ± 0.82
PGB_x[c]	200 μg	7	14.1 ± 0.97

[a]Hearts obtained from normal male rats (220-250 g).
[b]Physiological saline solution (0.9% NaCl), 0.2 ml.
[c]PGB_x calculated and expressed as the sodium salt. Hearts were perfused for a total of 20 minutes with control buffer prior to administration of the drug.
[d]Drugs were administered in a volume of 0.2 ml of PSS, followed by 0.5 ml of buffer as a wash to clear the cannula.
[e]Number of hearts in each group
[f]$$\%\ \text{Phosphorylase } \underline{a} = \frac{\text{Cori units of phosphorylase } \underline{a}}{\text{Total Cori units of phosphorylase } (\underline{a}+\underline{b})}$$

ANALYSIS OF VARIANCE SUMMARY TABLE[a]

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between groups (Dose)	2	0.86	0.43	0.73	0.494
Within groups (Error)	17	100.69	5.92		
TOTAL	19	101.55			

[a]$P \leq 0.05$ was considered significant.

TABLE 3-2

EFFECTS OF BOLUS ADMINISTRATION OF PGB_x ON GLYCOGEN CONTENT OF ISOLATED PERFUSED RAT HEARTS[a]

Drug	Dose[d]	N[e]	Glycogen (μM/g)[f]
PSS[b]	-	6	17.6 ± 1.6
PGB_x[c]	20 μg	7	15.5 ± 1.5
PGB_x[c]	200 μg	7	15.0 ± 1.0

[a]Hearts obtained from normal male rats (220-250 g).
[b]Physiological saline solution (0.9% NaCl), 0.2 ml.
[c]PGB_x calculated and expressed as the sodium salt. Hearts were perfused for a total of 20 minutes with control buffer prior to administration of the drug.
[d]Drugs were administered in a volume of 0.2 ml of PSS, followed by 0.5 ml of buffer as a wash to clear the cannula.
[e]Number of hearts in each group.
[f]Expressed per gram of tissue (wet weight).

ANALYSIS OF VARIANCE SUMMARY TABLE[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between groups (Dose)	2	24.49	12.24	0.95	0.406
Within groups (Error)	17	219.12	12.88		
TOTAL	19	243.6.			

[a]P<0.05 was considered significant.

TABLE 3-3

EFFECTS OF BOLUS ADMINISTRATION OF PGB_x ON ISOMETRIC SYSTOLIC TENSION IN ISOLATED PERFUSED RAT HEARTS[a]

Drug	Dose	N[e]	Pre	Post	Δ	$\frac{Post}{Pre} \times 100$
PSS[b]	-	6	16.3 ± 0.65	17.2 ± 0.73	0.9 ± 0.2	105.3 ± 1.16
PGB_x[c]	20 μg	7	13.9 ± 1.04	14.0 ± 0.09	0.8 ± 0.4	101.1 ± 3.53
PGB_x[c]	200 μg	7	15.9 ± 0.52	16.4 ± 0.59	0.5 ± 0.2	103.0 ± 1.51

[a]Hearts obtained from normal male rats (220-250 g).
[b]Physiological saline solution (0.9% NaCl), 0.2ml.
[c]PGB_x calculated and expressed as the sodium salt. Hearts were perfused for a total of 20 minutes with control buffer prior to administration of the drug.
[d]Drugs were administered in a volume of 0.2 ml of PSS, followed by 0.5 ml of buffer as a wash to clear the cannula.
[e]Number of hearts in each group.

ANALYSIS OF VARIANCE SUMMARY TABLE-A[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between groups (Dose)	2	57.63	28.81	0.74	0.491
Within groups (Error)	17	660.56	38.85		
TOTAL	19	718.19			

[a]$\frac{Post}{Pre} \times 100$ column, $P \leq 0.05$ was considered significant.

ANALYSIS OF VARIANCE SUMMARY TABLE-B[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between groups (Dose)	2	0.55	0.27	0.44	0.65
Within groups (Error)	17	10.70	0.62		
TOTAL	19	11.25			

[a]Δ column, $P \leq 0.05$ was considered significant.

TABLE 3-4

EFFECTS OF ISOPROTERENOL AND PGB_x ON PHOSPHORYLASE ACTIVITY IN ISOLATED PERFUSED RAT HEARTS[a]

Treatment[c]	Isoproterenol (ng)[b]					
	0	2.5	5.0	10.0	30.0	100.0
None	14.8 ± 2.24 (6)	23.3 ± 2.66 (9)	19.2 ± 4.99 (8)	41.4 ± 4.73 (5)	57.3 ± 5.22 (5)	70.7 ± 2.57 (5)
PGB_x (100 ng/ml)	15.1 ± 1.38 (6)	22.0 ± 2.31 (9)	26.3 ± 2.34 (8)	41.3 ± 6.40 (5)	63.8 ± 4.95 (5)	60.3 ± 2.57 (5)

[a]Hearts obtained from normal male rats (220-250 g). Figures in parenthesis indicate the number of hearts in each group.

$$\%\ \text{phosphorylase } \underline{a} = \frac{\text{Cori units of phosphorylase } \underline{a}}{\text{Total Cori units of phosphorylase } (\underline{a+b})} \times 100$$ plus or minus the standard error of the mean for each group.

[b]Calculated and expressed as the free base, and administered as a bolus in 0.2 ml PSS followed by a 0.5 ml KR buffer wash.

[c]Hearts were equilibrated for 15 minutes with drug-free KR buffer followed by an additional 15 minutes of perfusion with either drug-free or PGB_x-containing KR buffer before injection of isoproterenol. PGB_x was dissolved in the perfusion medium and its concentrations (100 ng/ml) was calculated and expressed as the sodium salt.

ANALYSIS OF VARIANCE SUMMARY TABLE[a]

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	1	10.80	10.80	0.13	0.719
Dose (Isoproterenol)	5	26,213.01	5,242.60	62.54	<0.001
Treatment x Dose	5	576.71	115.34	1.38	0.243
Within Cell	64	5,364.82	83.83		
TOTAL	75	32,165.34			

[a]$P \leq 0.05$ was considered significant

TABLE 3-5

EFFECTS OF ISOPROTERENOL AND PGB_x ON THE GLYCOGEN CONTENT OF ISOLATED PERFUSED RAT HEARTS[a]

Treatment[c]	Isoproterenol (ng)[b]					
	0	2.5	5.0	10.0	30.0	100.0
None	17.1 ± 2.16 (6)	17.4 ± 0.68 (9)	15.9 ± 1.13 (8)	18.9 ± 1.27 (5)	16.2 ± 1.33 (5)	15.5 ± 2.51 (5)
PGB_x (100 ng/ml)	17.0 ± 0.97 (6)	18.5 ± 1.07 (9)	15.2 ± 1.13 (8)	16.8 ± 1.81 (5)	15.8 ± 0.25 (5)	16.0 ± 0.71 (5)

[a]Hearts obtained from normal male rats (220-250 g). Figures in parenthesis indicate the number of hearts in each group. Glycogen content was calculated on a tissue wet weight basis and expressed as μM/g plus or minus the standard error of the mean for each group.

[b]Calculated and expressed as the free base, and administered as a bolus in 0.2 ml PSS followed by a 0.5 ml KR buffer wash.

[c]Hearts were equilibrated for 15 minutes with drug-free KR buffer followed by an additional 15 minutes of perfusion with either drug-free or PGB_x-containing KR buffer before injection of isoproterenol. PGB_x was dissolved in the perfusion medium and its concentration (100 ng/ml) was calculated and expressed as the sodium salt.

ANALYSIS OF VARIANCE SUMMARY TABLE[a]

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment (PGB_x)	1	0.42	0.46	0.04	0.842
Dose (Isoproterenol)	5	75.84	15.17	1.39	0.239
Treatment x Dose	5	18.94	3.79	0.35	0.880
Within Cell	64	697.44	10.90		
TOTAL	75	792.69			

[a]$P \leq 0.05$ was considered significant.

TABLE 3-6

EFFECTS OF ISOPROTERENOL AND PGB_x ON ISOMETRIC SYSTOLIC TENSION IN ISOLATED PERFUSED RAT HEARTS[a]

Treatment[c]	Isoproterenol (ng)[b]					
	0	2.5	5.0	10.0	30.0	100
None	102.9 ± 1.85 (6)	118.7 ± 3.10 (9)	115.7 ± 2.33 (8)	124.0 ± 1.73 (5)	130.6 ± 5.48 (5)	127.0 ± 2.3[illegible] (5)
PGB_x (100 ng/ml)	92.2 ± 8.45 (6)	118.6 ± 2.10 (9)	125.4 ± 5.81 (8)	129.9 ± 6.60 (5)	125.5 ± 2.55 (5)	128.9 ± 2.7[illegible] (5)

[a]Hearts obtained from normal male rats (220-250 g). Figures in parenthesis indicate the number of hearts in each group. The values represent $\frac{\text{Post-injection tension(g)}}{\text{Pre-injection tension(g)}} \times 100$ plus or minus the standard error of the mean for each group.

[b]Calculated and expressed as the free base, and administered as a bolus in 0.2 ml PSS followed by a 0.5 ml KR buffer wash.

[c]Hearts were equilibrated for 15 minutes with drug-free KR buffer followed by an additional 15 minutes of perfusion with either drug-free or PGB_x-containing KR buffer before injection of isoproterenol. PGB_x was dissolved in the perfusion medium and its concentration (100 ng/ml) was calculated and expressed as the sodium salt.

ANALYSIS OF VARIANCE SUMMARY TABLE[a]

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment (PGB_x)	1	8.56	8.56	0.07	0.792
Dose (Isoproterenol)	5	7,809.39	1,561.88	13.57	<0.001
Treatment x Dose	5	868.89	173.78	1.51	0.199
Within Cell	64	7,368.64	115.14		
TOTAL	75	16,055.48			

[a]$P \leq 0.05$ was considered significant.

PART IV

INTRODUCTION

Polis and Polis (1976) first demonstrated that PGB_x, a polymeric derivative of prostaglandin B_1, not to be confused with PGX (PGI_2 or prostacyclin) (Gryglwski *et al.*, 1976; Johnson *et al.*, 1976; Lefer *et al.*, 1978), had the ability to lower elevated blood glucose concentrations markedly in hereditary diabetic mice of the C57BL/KsJ strain (Jackson Laboratory, Bar Harbor, Maine) and to restore their abnormally high body weights towards normal. These investigators (Polis & Polis, 1979) reported, subsequently, that the hypoglycemic action of PGB_x was dose-related and that body weight and food intake of these diabetic mice also showed dose-dependency. As the dose of PGB_x administered to the animals was increased, the hypoglycemic effect became more pronounced, but food intake and body weight showed an inverse relationship to the quantity of drug which the mice received. The diabetic syndrome in the mice used by Polis and Polis (1976, 1979) in their studies was similar, in several ways, to that reported in humans, since the mice exhibited a diminished sensitivity to apparent normal concentrations of insulin, obesity, an elevated blood sugar and a diminished lifespan. Consequently, it has been suggested, that PGB_x may be of potential value in treating certain forms of human *diabetes mellitus* (Polis & Polis, 1976).

In the experiments reported herein, we have determined the actions of PGB_x on the glucose uptake and glycogen content of skeletal muscle of isolated rat diaphragms obtained from normal animals. These studies are the first of a series planned to investigate the metabolic effects of PGB_x on skeletal muscle in both normal and diabetic animals.

MATERIALS AND METHODS

Surgical & Incubation Methods

Wistar male albino rats (approximately 80-100 g) given Purina Lab Chow and water *ad libitum* were used throughout the course of this study. They were sacrificed by decapitation, each hemidiaphragm was carefully removed, rinsed in chilled physiological saline (PSS, 0.9% NaCl), blotted and weighed. The 25 ml Erlenmyer flasks in which the hemidiphragms were incubated individually, contained an initial volume of 2.0 ml of an incubation medium which had the following composition: 0.04M HEPES (N-2-hydroxyethyl piperazine-N-ethane sulfonic acid) neutralized to pH 7.2 with Na OH, 0.005M Mg Cl_2, 0.005M KCl, 0.103M NaCl, and 0.006M glucose. The final volume of the incubation medium in each flask was adjusted to 2.1 ml with PSS after the addition of PGB_x to the appropriate flasks. Each flask was gassed with 100% O_2, immediately following placement of a hemidiphragm into the flask, and it was closed tightly with a rubber stopper before being placed in a Dubnoff metabolic shaking incubator set at 37^o. The flasks were opened briefly at 30 minutes, regassed, immediately resealed and the incubation period was continued for a total of 60 minutes. The gassing procedure was omitted for those tissues incubated under room air. Control (blank) flasks-containing no tissues were carried through the incubation procedure for verification of their initial glucose content.

Analytical Methods

At the conclusion of the 60 minute incubation period, the flasks were removed from the incubator, inverted several times to recover and mix with the flask contents any condensate that had formed on the inside wall of the reaction

vessel, opened and placed in an ice bath. The tissues were removed with forceps, blotted on filter paper and immediately placed individually in tubes containing 1 ml of 30% warm KOH in preparation for extraction and measurement of their glycogen content according to the colorometric method of Montgomery (1957).

An 0.8 ml aliquot of the chilled incubation medium from each flask, including the blanks, was acidified with cold 0.625 N perchloric acid, and, subsequently, neutralized with 0.5M TRA-1.67M K_2CO_3 buffer to yield a final volume of 1.1 ml. Aliquots of the neutralized perchloric acid/incubation medium were analyzed for their glucose content according to the enzymatic method of Bergmeyer *et al* (1974).

Statistical Methods

The data were examined by analysis of variance (ANOVA) and by a *t* test designed to compare individual cells and groups of cells within the analysis of variance matrix (Snedecor & Cochran, 1967; Sokal & Rohlf, 1969; Winer, 1962).

Drugs

The PGB_x used in the experiments described in this section was obtained, prepared and stored as reported in Part I.

RESULTS

Effects of PGB_x on Glucose Uptake and Glycogen Content of Rat Hemidiaphragms Incubated Under 100% O_2

When PGB_x was added to reaction flasks in concentrations which ranged from 25-400 μg/flask (total μg/2.1 ml) and the tissues were incubated under an environment of 100% O_2 for exactly 60 minutes, a significant dose dependent decrease in glucose utilization was noted when the data was analyzed by analysis of variance (Table 4-1). When, however, the individual PGB_x-treated groups were compared to the control group within the ANOVA matrix, taking into account the variance within the experimental population, significant differences between groups were not observed at concentrations below 200 μg/flask. At the two higher dosages, 200 and 400 μg/flask, glucose utilization was significantly below ($P=0.021$ and 0.036 respectively) that found in the control (PGB_x-free) flasks (Table 4-1).

Although PGB_x had an effect on glucose utilization, it did not alter significantly the glycogen content of these same tissues (Table 4-2) over the entire dosage range. When compared individually with the control group, the concentrations of glycogen in the PGB_x-treated groups were found also to be the same.

Effects of PGB_x on Glucose Uptake and Glycogen Content of Rat Hemidiphragm Incubated Under Room Air

Tissues incubated under room air showed no response to PGB_x, over a dosage range of 100 to 400 μg/flask, with regard to glucose utilization (Table 4-2) and the glycogen content of the PGB_x-treated hemidiphragms did not differ from those which were not exposed to PGB_x.

DISCUSSION

In their studies with hereditary diabetic mice, Polis and Polis (1979) demonstrated that PGB_x, as a function of its dosage, lowered the concentration of blood glucose and reduced the body weight of such animals. They found also that the food intake of their diabetic mice decreased progressively as they increased the dosage of PGB_x. On the basis of this data alone, one might speculate that perhaps PGB_x caused anoxexia and that this contributed to the weight loss and reduction in blood glucose concentration in the same manner that dietary restrictions have helped control the disease process in humans with maturity-onset *diabetes mellitus* (MOD) (Mauer, 1979) and in insulin-resistant obese diabetic mice (Carnie & Smith, 1980). In the latter case, hyperglycemia was definitely related to increased food intake and dietary restriction resulted in a return towards normal blood glucose concentrations. It is interesting to note, however, that when PGB_x (20 μg/g) was used to treat normal mice for 5 weeks, their body weight and blood glucose concentrations were not altered significantly as a consequence of receiving the drug (Polis & Polis, 1979) That finding supports the view that beneficial effects reported by Polis and Polis (1979) were attributed to a unique, but yet undetermined, action of PGB_x rather than a non-specific PGB_x-induced reduction of food intake.

While Polis and Polis (1979) have suggested that PGB_x may enable cells in heredity diabetic mice to utilize insulin more efficiently, direct evidence to support their proposed mechanism is not yet available. Our data (Tables 4-1 & 4-3) obtained through study of "normal" tissue, did not reveal a direct PGB_x-induced enhancement of glucose uptake, but rather a significant decrease at higher PGB_x

concentrations. The ability of drugs to diminish glucose uptake in this preparation has reported previously by others. For example, high concentrations of halothane (Rosenberg *et al*, 1977) decreased glucose uptake, both in the presence and absence of insulin, and Haugaard *et al*, 1976, demonstrated that the inhibition of glucose uptake produced by epinephrine was almost equal to the epinephrine-induced decrease in the incorporation of ^{14}C glucose into glycogen in this preparation. The possibility that a high capacity for glycogen synthesis within a tissue is required in order for the inhibitory effect epinephrine to be apparent has been raised by Haugaard *et al* (1976).

In skeletal muscle, epinephrine has been shown, by activating phosphorylase, to elevate tissue concentrations of glucose-6-phosphate, a metabolite known to inhibit hexokinase preparations, and this may serve as one possible mechanism which glucose utilization and glycogen synthesis are required (Haugaard *et al*, 1976). However, the correlation between elevated glucose-6-phosphate and decreased glucose uptake did not hold in the case of insulin, where glucose-6-phosphate concentrations increased along with glucose utilization, and with lithium ions which increased glucose uptake while simultaneously decreasing glucose-6-phosphate (Haugaard *et al*, 1974). While the effects of PGB_x on glucose 6-phosphate and related enzyme systems has not been determined in rat diaphragm, it has been shown to elevate glucose-6-phosphate concentrations in isolated perfused rat hearts (Aronson & Guerrero, 1979). It is possible that the PGB_x-induced inhibition of glucose uptake which we observed in isolated rat diaphragm may occur by a mechanism related to glucose-6-phosphate concentrations and hexokinase activity in the tissue or perhaps as a consequence of an inhibition of mitochondrial function in this tissue. Further experiments are required, however, in order to explore fully these possibilities.

The question of how PGB_x promotes glucose uptake in diabetic mice and its possible relationship to insulin and insulin receptors in such animals remains unanswered. It is important to recognize, however, that insulin resistance is found not only in hereditary diabetic mice but also has been reported by numerous investigators to occur in other situations, for example, following injury and burns (Chaudry *et al*, 1974; Chaudry *et al*, 1975; Frayn *et al*, 1978; Nelson & Filkins, 1979; Ryan *et al*, 1974a,b; Ryan *et al*, 1977; Turnisky *et al*, 1977; Vigas & Nemeth, 1972; Wolfe *et al*, 1976). Studies by Ryan *et al* (1977) and Chaudry *et al* (1974, 1975) suggested that the decreased effect of insulin on glucose utilization which they observed was not dependent on concurrent alterations in plasma concentrations of adrenal steroids, catecholamines or glucogen. While insulin resistance has been clearly demonstrated *in vitro* following hemorrhage (Chaudry *et al*, 1974, 1975; Ryan *et al*, 1974b, 1977) the lack of maintenance in isolated tissues of the insulin resistance observed *in vivo* caused Nelson and Filkens (1979) to speculate concerning the possible existance of a substance that interfered with the action of insulin under such circumstances. No attempt was made, however, to state the source and nature of their proposed endogenous inhibitory substance. It would, therefore, be of interest to conduct studies similar to those of Nelson and Filkens (1979) using the Noble-Collip method of inducing traumatic injury (Noble & Collip, 1942) in order to see whether PGB_x might have a protective action against the trauma induced insulin resistance and hyperglycemia reported to have been caused by such treatment.

Although there are numerous papers in the literature which provide evidence to support the view that insulin promotes glucose uptake by first interacting with specific receptors on the plasma membrane of the cell and that the insulin is bound to these receptor sites (Jarrett, 1979; Levine *et al*, 1965;

Stadie *et al*, 1949; Freychlet *et al*, 1971; Hammond *et al*, 1972), our studies did not demonstrate an insulin-like hypoglycemic action for PGB_x in tissues from normal animals. Consequently, we cannot postulate the existance, in skeletal muscle of rat diaphragm from normal animals, of a receptor site, sensitive to PGB_x, that either mimics the action of insulin or works in concert with it.

Jarett (1979) has pointed out that it is still not known with certainty whether only one type of insulin receptor exists or whether there are several, perhaps due to an interconversion from one form to another, as suggested by the work of Olefsky and Chong (1978). One cannot rule out the possibility, on the basis of existing evidence, that perhaps, in diabetic mice like those used by Polis and Polis (1976, 1979), PGB_x interacted favorably with either "altered" receptors somehow rendered insensitive to insulin or by some other unique, but, as yet, unidentified action.

While the actual mechanism by which the number and the affinity of insulin receptors remains unsettled, self-regulation of these receptors does not occur when protein synthesis is inhibited, or, for example, when ATP levels are reduced, Jarett (1979). The relationship between membrane degradation of receptor-bound insulin and the ability of insulin to cause a biological effect has also been pointed out by Jarett (1979), and one might reasonably ask whether PGB_x has any influence on such a system.

In studies with human patients having nonketotic *diabetes mellitus*, there has been demonstrated a reduction in the number of insulin receptors on circulating monocytes. The 50% reduction that occurred took place without a change in receptor affinity (Olefsky & Reaven, 1974, 1976a,b). In such patients, sulfonylurea treatment increased the receptor population and decreased the level of

hyperglycemia (Olefsky & Reaven, 1976). Once again, it is not known whether PGB_x can exert a similar action, and it is a possibility that deserves further consideration.

Our studies with PGB_x and the isolated rat diaphragm represent only a first step towards better understanding how this interesting substance can influence carbohydrate metabolism in skeletal muscle and then have pointed out clearly to us the need for future research to focus in on the various sites where PGB_x may be exerting its unique effects.

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TABLE 4-1

Effects of PGB_x on Glucose Uptake by Isolated Rat Diaphragms Incubated Under 100% O_2[a]

Amount of PGB_x in the Incubation Flask[b]					
0	25 μg	50 μg	100 μg	200 μg	400 μg
19.59 ± 1.94 (16)[c]	24.05 ± 1.76 (10)[c]	21.74 ± 3.19 (10)[c]	20.05 ± 1.76 (12)[c]	13.20 ± 1.21 (10)[c]	14.11 ± 1.34 (12)[c]

[a]Tissue obtained from normal male rats weighing 80-100 grams. Glucose uptake expressed as μM/g (wet weight)/hr ± S.E.M.
[b]Total quantity of PGB_x (Preparation #25) calculated and expressed as the sodium salt, in a final volume of 2.1 ml.
[c]Figures in parenthesis signify the number of hemidiaphragms in each group.

Analysis of Variance Summary Table[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P[a]
Between Groups (Dose)	5	967.25	193.45	4.31	0.002
Within Groups (Error)	64	2875.35	44.93		
Total	69	3842.60			

[a]$P \leq 0.05$ was considered significant.

TABLE 4-2

Effects of PGB_x on the Glycogen Content of Isolated Rat Diaphragms Incubated Under $100\%\ O_2$[a]

Amount of PGB_x in the Incubation Flask[b]					
0	25 g	50 g	100 g	200 g	400 g
24.90 ± 1.05 (16)[c]	26.37 ± 1.88 (10)[c]	24.93 ± 1.22 (10)[c]	25.86 ± 0.95 (12)[c]	26.08 ± 1.20 (10)[c]	27.36 ± 0.53 (12)[c]

[a]Tissue obtained from normal male rats weighing 80-100 grams. Glycogen content expressed as μM/g (wet weight) ± S.E.M.

[b]Total quantity of PGB_x (Preparation #25), calculated and expressed as the sodium salt, in a final volume of 2.1 ml.

[c]Figures in parenthesis signify the number of hemidiaphragms in each group.

Analysis of Variance Summary Table[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P[a]
Between Groups (Dose)	5	53.32	10.66	0.68	0.642
Within Groups (Error)	64	1007.22	15.74		
Total	69	1060.54			

[a]$P \leq 0.05$ was considered significant.

TABLE 4-3

Effects of PGB_x on Glucose Uptake by Isolated Rat Diaphragms Incubated Under Room Air[a]

Amount of PGB_x in the Incubation Flask[b]		
0	100 μg	400 μg
16.97 ± 3.21 (10)[c]	13.70 ± 2.74 (10)[c]	11.62 ± 1.60 (10)[c]

[a]Tissue obtained from normal male rats weighing 80-100 grams. Glucose uptake expressed as μM/g (wet weight)/hr ± S.E.M.
[b]Total quantity of PGB_x (Preparation #25), calculated and expressed as the sodium salt in a final volume of 2.1 ml.
[c]Figures in parenthesis signify the number of hemidiaphragms in each group.

Analysis of Variance Summary Table[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between Groups (Dose)	2	144.82	72.41	1.07	0.358
Within Groups (Error)	27	1834.98	67.96		
Total	29	1979.80			

[a]$P \leq 0.05$ was considered significant.

TABLE 4-4

Effects of PGB_x on the Glycogen Content of Isolated Rat Diaphragms Incubated Under Room Air[a]

Amount of PGB_x in the Incubation Flask[b]		
0	100 μg	400 μg
21.58 ± 1.78 (10)[c]	24.24 ± 2.09 (10)[c]	17.30 ± 1.26 (10)[c]

[a]Tissue obtained from normal male rats weighing 80-100 grams. Glycogen content expressed as μM/g (wet weight) ± S.E.M.
[b]Total quantity of PGB_x (Preparation #25), calculated and expressed as the sodium salt in a final volume of 2.1 ml.
[c]Figures in parenthesis signify the number of hemidiaphragms in each group.

Analysis of Variance Summary Table[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between Groups (Dose)	2	245.33	122.66	4.04	0.029
Within Groups (Error)	27	820.15	30.38		
Total	29	1065.48			

[a]$P \leq 0.05$ was considered significant.